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(54) **METHOD FOR COUNTING LIVING CELLS**

(57) The present invention provides a method for rapidly and sensitively counting living cells in a sample by serially diluting the sample containing living cells with a growth medium for living cells, culturing the resulting

dilution, and then measuring an intracellular ATP level in the dilution using a luciferin-luciferase luminescent reaction; an assay kit used for the above method; and a living cell counting device for carrying out the above method.

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## Description

## TECHNICAL FIELD

- 5 [0001] The present invention relates to a method for rapidly and sensitively counting living cells present in a sample, an assay reagent kit, and a living cell counting device.

## BACKGROUND ART

- 10 [0002] In various industrial fields, clinical medicine, basic research and the like, cell counting is broadly carried out for determining the number of living cells in a sample liquid. Particularly, in the food, pharmaceutical and cosmetic industries, it is very important to control the number of living cells, including microorganisms, present in industrial water, raw materials, intermediates or end products. In this case, a rapid determination is required rather than more perfect cell counting.

- 15 [0003] To determine the number of living cells in a sample, in general, a known plate culture method is conventionally carried out, in which living cells in the sample are cultured on a standard agar medium for a long time, followed by the detection of colonies formed thereon. The sample may be used for the culture as such or after trapping the living cells on a simple filter. See "Guidelines for Food Sanitation Inspection - Microorganisms" by Environmental Health Bureau, Ministry of Health and Welfare (1990) pp.70-77. In addition, other known methods for determining the number of living  
20 cells in a sample include the most probable number method (also called MPN method or Serial dilution method), which is now employed in Japan as an official method for analyzing the quality of pharmaceuticals and cosmetics etc. (The Japanese Pharmacopoeia, Thirteenth Edition, published by Hirokawa Publishing Co. (1996) p.B-403 to p.B-421) In the most probable number method, the number of living cells is determined by serially diluting a sample; inoculating an aliquot of each dilution into a liquid medium, followed by culture for a long time; determining the presence of living  
25 cells grown in the medium by its turbidity; and then calculating the number of living cells in the sample as the most probable number, based on a statistical probability theory.

- [0004] In the plate culture method, the number of living cells is determined by applying a sample onto a solid plate medium such as agar medium, culturing it for about 2 to 7 days, and then counting colonies grown on the plate medium. Accordingly, the plate culture method has a disadvantage of permitting no rapid determination. The most probable  
30 number method also has a disadvantage of requiring a long culture period because the presence of living cells grown in the medium is determined by turbidimetry.

- [0005] Recently, a luminescent reaction based on a luciferin-luciferase reaction is utilized for counting living cells (e. g., De Luca, M. & McElroy, W.D. (1978) Methods Enzymol. 57:3-15; Stanley, P.E. (1986) Methods Enzymol. 133:14-22). In the cell counting method based on the luciferin-luciferase luminescent reaction, the number of living cells is deter-  
35 mined by extracting ATP from living cells, allowing the extracted ATP to produce luminescence through the luciferin-luciferase luminescent reaction, measuring an ATP level from the intensity of luminescence, and then calculating the number of living cells from the measured ATP level and an ATP level per living cell.

- [0006] By way of example for cell counting using a bioluminescent reagent through the luciferin-luciferase luminescent reaction, Figure 1 shows the calibration curve prepared using CheckLite 250 Plus (Kikkoman Corp.) that converts  
40 the luminescence intensity into the number of *Escherichia coli* cells. As shown in Figure 1, the lower detection limit for *Escherichia coli* is  $10^3$  CFU (unit of living cell)/ml. On the other hand, the ATP content per bacterial cell is reported to be almost constant among bacterial cell types (Kodaka et al., Japanese Journal of Food Microbiology, 13:29-34 (1996)). When the bioluminescence method is applied for bacterial cells in general, it is therefore possible to determine the number of living cells using the calibration curve mentioned above (i.e., Figure 1), but the detection limit in this case  
45 is also  $10^3$  CFU/ml. Namely, the above method involves a problem that it is theoretically impossible to determine the number of living cells below the lower detection limit.

- [0007] Further, when used for cell counting, the above method involves another problem that if ATP other than intracellular ATP (hereinafter, background ATP) is present at a high level in a sample, the intracellular ATP is buried in the background ATP, leading to a decrease in detectability. The decomposition of background ATP may be carried out  
50 using a reagent containing ATPase as an active ingredient. However, such a treatment cannot overcome the problem because it might results in insufficient decomposition of ATP.

- [0008] With regard to a sample below the lower detection limit or containing background ATP at a high level, such a sample may be cultured for a short time to grow living cells to a detectable level. This makes the living cells detectable, but it is still impossible to determine the number of living cells in the sample.

- 55 [0009] The object of the present invention is to provide a method for counting living cells present in a sample with high accuracy in a short time, an assay reagent kit used for the method, and a living cell counting device for carrying out the method.

## DISCLOSURE OF THE INVENTION

[0010] Our research efforts were directed to overcoming the above problems, and we have found that the number of living cells in a sample can be determined with high accuracy in a short time by serially diluting the sample with a growth medium for living cells, culturing the resulting dilution, and then determining the presence of living cells in the cultured dilution using the luciferin-luciferase luminescent reaction, thereby finally completing the invention.

[0011] Namely, the present invention provides the following embodiments (1) to (7).

(1) A method for counting living cells in a sample, which comprises the following steps (i) to (iv):

- (i) a first step for serially diluting the sample with a diluent solution;
- (ii) a second step for culturing each dilution from the first step;
- (iii) a third step for detecting intracellular ATP in each of the cultured dilutions from the second step; and
- (iv) a fourth step for calculating the number of living cells in the sample, based on the results of intracellular ATP detection from the third step.

(2) The method according to (1) above, wherein the diluent solution used in the first step is a growth medium for living cells.

(3) The method according to (1) above, wherein the diluent solution used in the first step is a growth medium for living cells which has been subjected to ATP decomposition.

(4) The method according to (1) above, wherein ATP present in the dilution, excluding the intracellular ATP, is decomposed during or after the culture in the second step.

(5) The method according to (1) above, wherein the intracellular ATP is detected in the third step using a luciferin-luciferase luminescent reaction.

(6) An assay reagent kit used for the method according to (1) above, which comprises the following components (a) to (c):

- (a) a diluent solution,
- (b) an ATP-extracting reagent, and
- (c) an ATP-detecting reagent.

(7) A living cell counting device used for the method according to (1) above, which comprises a device for sample dilution, a culture device for cell growth and a device for ATP detection.

(8) The living cell counting device according to (7) above, wherein the device for ATP detection measures the intensity of luminescence produced through a luciferin-luciferase luminescent reaction.

[0012] This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 10-333979, which is a priority document of the present application.

## BRIEF DESCRIPTION OF DRAWING

[0013] Figure 1 shows the calibration curve that was mentioned in Background Art and used in comparative method 2 of Examples 1, 2 and 3.

## BEST MODE FOR CARRYING OUT THE INVENTION

[0014] As used herein, the term "living cell" means any cell capable of growing. Such living cells include, but are not limited to, animal cells, plant cells and microorganisms (e.g., bacteria, yeast, filamentous fungi), and may be selected depending on the application purpose of the method according to the present invention. They may be used alone or by mixture.

[0015] A "sample" may be any sample suspected of containing the above living cells, including foods and drinks, pharmaceuticals, cosmetics, sea water, river water, industrial water, sewage, soil, urine, feces, blood, sputum, pus, and living cell cultures. The sample is used in liquid form for assay. When the sample contains solids or is viscous, it

is desirably suspended in an appropriate solvent to give a solution sample. Such a solvent includes distilled water, physiological saline, phosphate buffer, Tris buffer and sodium acetate buffer.

[0016] In the first step of the present invention, a collected sample is subjected to serial dilution using a diluent solution. Illustrative examples of a diluent solution include, in addition to the growth medium for living cells mentioned above, distilled water, physiological saline, phosphate buffer, Tris buffer and sodium acetate buffer. In particular, the growth medium for living cells to be assayed is preferably used as a diluent solution. As used herein, the term "growth medium for living cells" means a solution that permits the proliferation and growth of living cells and that contains nutrient components necessary for their proliferation and growth. Any growth medium suitable for target living cells may be selected as a growth medium for living cells used in the present invention. Once target living cells are specified, those skilled in the art will readily select a suitable growth medium. For example, a suitable growth medium may be Dulbecco's MEM medium for animal cell growth, nutrient broth medium for bacterial cell growth, and dextrose peptone broth for yeast cell growth.

[0017] The serial dilution means a series of dilution cycles repeated in a manner that a sample is diluted with a diluent solution to give a first dilution, which is then similarly diluted with a diluent solution to give a second dilution. The serial dilution can provide a stepwise increase in dilution factor. Further, any dilution factor may be selected without any particular limitation. However, an extremely high dilution factor may produce less accurate dilution, while an extremely low dilution factor may provide an increase in the number of subsequent dilution cycles. Such an extremely high or low dilution factor is therefore not preferable. In the assay of the present invention in which the order-of-magnitude accuracy is required, it is preferable to serially dilute a sample by 10-fold for ease of calculation of cell count. In this case, for example, 1 part of a sample and 9 parts of a diluent solution are combined to give a 10-fold dilution. This dilution procedure can be repeated to give a series of 10-fold dilutions including 10-fold dilution, 100-fold dilution, and 1,000-fold dilution etc.

[0018] In the second step of the present invention, each dilution from the first step is cultured. As used herein, the term "culture" or "culturing" means that living cells are allowed to be grown experimentally in a growth medium for living cells under controlled external conditions including temperature, oxygen concentration, and culture period etc. Once target living cells and a growth medium to be used are specified, those skilled in the art will readily determine external conditions suitable for cell culture. In the case of bacterial cells, for example, a dilution containing bacterial cells diluted with a growth medium for living cells in a test tube is preferably cultured at 30 °C for 6 hours while shaking at 100 rpm. Depending on target living cells, culture conditions may be selected from the following ranges as appropriate: a culture temperature from 5 to 80 °C, a shaking condition from static culture to shaking culture at 500 rpm, and a culture period from 1 to 120 hours.

[0019] In the present invention, background ATP present in a dilution can cause a decrease in detectability because the presence of living cells is determined based on their intracellular ATP level. Thus, it is desirable to decompose the background ATP present in a diluent solution and a dilution or cultured dilution, prior to the extraction of intracellular ATP from the living cells. To decompose the background ATP, for example, a reagent containing ATPase as an active ingredient (hereinafter, ATP-eliminating agent) may be used. Illustrative examples of ATPase include adenosine phosphate deaminase, apyrase, alkaline phosphatase, acid phosphatase, hexokinase, and phosphodiesterase. These enzymes may be used alone or in combination. After decomposing the background ATP, it is desirable to remove or deactivate the ATP-eliminating agent in a dilution. The ATP-eliminating agent may also be deactivated by treatment with its inhibitor, including cofomycin against adenosine phosphate deaminase, actinomycin D against alkaline phosphatase, xylose against hexokinase, and xanthine against phosphodiesterase. In addition to these inhibitors, appropriate physical conditions (e.g., heat, pH, ionic strength) may also be used for deactivation.

[0020] In the present invention, the decomposition of background ATP may be achieved by a method in which a diluent solution pre-treated with the ATP-eliminating agent to decompose ATP is used as a diluent solution for the first step, or by a method in which the background ATP is decomposed with the ATP-eliminating agent during or after the culture in the second step. These methods may be used in combination.

[0021] The steps of the present invention may be combined with cell collection using a filter in order to further improve the detectability. For example, each dilution from the first step may be filtrated through a filter to collect cells, which may then be cultured in the second step. Alternatively, each cultured dilution from the second step may be filtrated through a filter to collect cells, which may then be used for intracellular ATP detection in the third step.

[0022] To detect intracellular ATP in the third step of the present invention, the intracellular ATP is first extracted from living cells present in a cultured dilution or an aliquot taken therefrom, and the extracted ATP is then assayed for its level. Secondly, the ATP level thus obtained is compared with an ATP level detected in a control sample without living cell (blank value) to determine the presence of living cells. For example, in the below-mentioned ATP detection based on the luciferin-luciferase luminescent reaction method, a sample can be identified to contain living cells when luminescence produced from the sample has an intensity of at least 1.5 times higher than the blank value. However, such a numerical value may be determined appropriately, depending on the types of living cell and diluent solution, and/or how the sample and control are prepared.

[0023] The intracellular ATP may be extracted from living cells by heat extraction or by treatment with an ATP-extracting reagent. In the former, the dilution is heated at 50 to 100 °C for 1 to 60 minutes to release the intracellular ATP out of the cells. In the latter, the ATP-extracting reagent is added to the dilution, followed by extraction. As an ATP-extracting reagent, a known reagent may be used, including lysozyme, a mixture of ethanol and ammonia, methanol, ethanol, surfactants (e.g., benzethonium chloride, benzalkonium chloride, Triton X-100), trichloroacetic acid, and perchloric acid. For example, an ATP-extracting reagent packaged with CheckLite 250 Plus commercially available from Kikkoman Corp. may be used. Next, the extracted ATP may be detected by various known ATP detection methods. For example, an ATP-detecting reagent may be added to the dilution after ATP extraction. Any reagent for detecting ATP can be used for this purpose. Specifically, a reagent for detecting ATP based on the luciferin-luciferase luminescent reaction method may be employed. Luciferin and luciferase used in the present invention may be those derived from fireflies such as Genji-firefly (*Luciola cruciata*), Heike-firefly (*Luciola lateralis*), American firefly (*Photinus pyralis*), and glowworm.

[0024] In the present invention, luciferase may be a naturally occurring luciferase purified from luminous tissue of organisms mentioned above, a genetically engineered wild-type luciferase, or a mutant luciferase modified by one or more amino acid addition, deletion and substitution in the amino acid sequence for the wild-type luciferase.

[0025] In the luciferin-luciferase luminescent reaction method, an ATP-detecting reagent comprising luciferin, luciferase and magnesium (hereinafter, bioluminescent reagent) is first added to the dilution after ATP extraction. Since luminescence is produced through the reaction between the bioluminescent reagent and ATP, its intensity is measured using a luminometer and the like. As a bioluminescent reagent, a bioluminescent reagent packaged with a commercially available reagent, for example, CheckLite 250 Plus (Kikkoman Corp.) may be used. Instead of the above luciferin-luciferase luminescent reaction method, a bioluminescence method modified to include pyruvate orthophosphate dikinase (hereinafter, PPDK luminescence method) can be used in order to further improve the detectability (Japanese Patent Laid-Open Publication No. 9-234099).

[0026] In the fourth step, the number of living cells in the original sample is determined based on the detected intracellular ATP level in each cultured dilution. Namely, the number of living cells in the sample and/or cell count with order-of-magnitude accuracy can be calculated from the intensity of luminescence produced, the ATP level obtained therefrom, and the dilution factor used. Simultaneously, a more accurate cell count for the sample may also be calculated using the most probable number table.

[0027] An assay reagent kit for counting living cells comprises the diluent solution, ATP-extracting reagent and ATP-detecting reagent mentioned above. ATP-extracting and -detecting reagents may be a commercially available reagent for detecting intracellular ATP based on the luciferin-luciferase luminescent reaction method, for example, CheckLite 250 Plus (Kikkoman Corp.). When the decomposition of background ATP is required for detection with high detectability, the background ATP is decomposed as described above, for example, using a CheckLite ATP Eliminating kit commercially available from Kikkoman Corp., prior to addition of the ATP-extracting agent. The kit may comprise other reagents, in addition to the above diluent solution and reagents. Examples of other reagents include an ATP-eliminating agent or the like.

[0028] Further, the living cell counting device according to the present invention, which is used for carrying out the above cell counting method, comprises a device for sample dilution, a culture device for cell growth and a device for ATP detection. The device for sample dilution is used for the serial dilution of a sample in test tubes. A commercially available dilution device, such as System Diluter (distributed by Gunze Sangyo, Inc.), may also be used for this purpose. The dilution device can automatically select any desired dilution condition, including diluent solution volume and serial dilution factor, under the control of a program stored therein. In turn, the culture device for cell growth is used for culturing living cells present in a dilution. Preferably, it can comprise a thermostat and a shaker.

[0029] The device for ATP detection comprises an extractor for extracting intracellular ATP and a detector for detecting the extracted ATP. The extractor can automatically extract intracellular ATP at room temperature by adding a predetermined amount of the ATP-extracting agent to a test tube containing the cultured dilution. The detector may be, for example, a luminometer for detecting bioluminescence produced through the luciferin-luciferase luminescent reaction. In this case, a predetermined amount of a luciferin-luciferase luminescent reagent is added to a test tube after ATP extraction to measure the intensity of luminescence produced. A suitable ATP detector, which can be used in the present invention, includes a commercially available detector, Lumitester K-100 (distributed by Seishin Corp.).

[0030] The number of living cells in the original sample is calculated from the resulting ATP level in the detection. The living cell counting device of the present invention may incorporate an arithmetic unit having the following calculation program:

- (1) a program which calculates cell count from ATP levels and dilution conditions; or
- (2) a program which calculates cell count from ATP levels and dilution conditions using the most probable number method based on a statistical probability theory. In addition, the device may also have additional functions including storage, backup and printout of various data.

[0031] The device for sample dilution, culture device for cell growth and device for ATP detection according to the present invention may be functionally connected with one another to provide the living cell counting device. Preferably, these three devices may be integrated into a unit system, such that the number of living cells in a sample can be determined automatically by just loading the sample in the system.

## EXAMPLES

[0032] The present invention will be further described in the following examples. These examples are provided for illustrative purposes only, and are not intended to limit the scope of the invention.

### Example 1: Determination of Living Cell Counts using Standard Bacterial Strains

[0033] As standard bacterial strains, *Escherichia coli* ATCC 25922 (hereinafter, Strain E) and *Bacillus subtilis* ATCC 9372 (hereinafter, Strain B) were used. Each of these two standard bacterial strains was inoculated into 3 ml of nutrient broth liquid medium and cultured overnight at 35 °C. Each resulting culture was appropriately diluted with sterilized physiological saline and used as a sample for the living cell counting mentioned below. A sample containing more living cells was designated Sample 1, while a sample containing less living cells was designated Sample 2.

#### (1) Inventive Method

[0034] 10 ml of ATP-eliminating agent such as CheckLite ATP Eliminating Kit (Kikkoman Corp.), which had been sterilized by filtration, was added to 90 ml of autoclaved fluid thioglycolate medium II (Eiken Chemical Co., Ltd.) to give a culture medium for living cells containing no ATP, which was then used as a diluent solution. 1 ml of Sample 1 was added to and stirred well with 9 ml of the diluent solution to produce a 10-fold dilution. Next, 1 ml of the 10-fold dilution was added to and stirred with 9 ml of the diluent solution to produce a 100-fold dilution. Finally, the same procedure was repeated to give a series of 10-fold dilutions, including 10-fold, 100-fold, 1,000-fold, 10,000-fold or more dilutions. Each dilution thus obtained was cultured at 35 °C for 6 hours. After culturing, 0.1 ml of each cultured dilution was transferred to a cuvette, followed by intracellular ATP extraction and bioluminescent reaction using CheckLite 250 Plus (Kikkoman Corp.). The intensity of bioluminescence resulting from the intracellular ATP was measured using a Lumitester K-100 (distributed by Kikkoman Corp.). The intensity of luminescence produced from the diluent solution alone (0.1 ml) was used as a blank value. When luminescence produced from a cultured dilution had an intensity of at least 1.5 times higher than the blank value, the cultured dilution was identified to contain living cells. Out of the dilutions identified to contain living cells, the dilution with a maximum dilution factor was chosen to calculate living cell count for the original sample from the dilution factor value.

#### (2) Comparative Method 1

[0035] Sterilized physiological saline was used as a diluent solution. Each sample was serially diluted to give a series of 10-fold dilutions. 100 µl of each dilution was applied on a plate count agar (Eiken Chemical Co., Ltd.) in a plastic schale and then cultured at 35 °C for 2 days. Colonies formed on the plate were counted to calculate living cell count for the original sample from the dilution factor value.

#### (3) Comparative Method 2

[0036] 1.0 ml of each sample was transferred to a cuvette and reacted with 0.1 ml of ATP-eliminating agent at room temperature for 30 minutes. 0.1 ml of the reacted sample was transferred to a cuvette, followed by intracellular ATP detection using CheckLite 250 Plus (Kikkoman Corp.) and a Lumitester K-100 (distributed by Kikkoman Corp.). The measured intensity of luminescence resulting from the intracellular ATP was converted into living cell count using the calibration curve shown in Figure 1.

#### (4) Results

##### [0037]

##### (a) In the case of Strain E:

(i) The results obtained by the inventive method are shown in Table 1.

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Table 1

Dilution factor	Sample 1		Sample 2	
	Intensity (RLU)	Growth	Intensity (RLU)	Growth
10	284404	Yes	3095	Yes
100	27898	Yes	255	Yes
1,000	2467	Yes	10	No
10,000	366	Yes	11	No
100,000	10	No	10	No
1,000,000	9	No	9	No
Blank value	10	No	10	No
Living cell count for Sample 1 (order of magnitude): $10^4$ CFU/ml				
Living cell count for Sample 2 (order of magnitude): $10^2$ CFU/ml				

(ii) The results obtained by comparative method 1 are shown in Table 2.

Table 2

Sample	Living cell count
Sample 1	$4.5 \times 10^4$ CFU/ml
Sample 2	$4.5 \times 10^2$ CFU/ml

(iii) The results obtained by comparative method 2 are shown in Table 3.

Table 3

Sample	Intensity	Living cell count
Sample 1	130 RLU	$1.8 \times 10^4$ CFU/ml
Sample 2	4 RLU	uncountable
Blank	5 RLU	

(b) In the case of Strain B:

(i) The results obtained by the inventive method are shown in Table 4.

Table 4

Dilution factor	Sample 1		Sample 2	
	Intensity (RLU)	Growth	Intensity (RLU)	Growth
10	77422	Yes	306	Yes
100	2249	Yes	53	Yes
1,000	181	Yes	12	No
10,000	22	Yes	11	No
100,000	11	No	9	No
1,000,000	9	No	10	No
Blank value	10	No	10	No
Living cell count for Sample 1 (order of magnitude): $10^4$ CFU/ml				
Living cell count for Sample 2 (order of magnitude): $10^2$ CFU/ml				

(ii) The results obtained by comparative method 1 are shown in Table 5.

Table 5

Sample	Living cell count
Sample 1	$4.4 \times 10^4$ CFU/ml
Sample 2	$4.4 \times 10^2$ CFU/ml

(iii) The results obtained by comparative method 2 are shown in Table 6.

Table 6

Sample	Intensity	Living cell count
Sample 1	560 RLU	$1.8 \times 10^4$ CFU/ml
Sample 2	6 RLU	uncountable
Blank	5 RLU	

[0038] Tables 1, 2, 4 and 5 showed that all living cell counts for Samples 1 and 2 determined by the inventive method and comparative method 1 were of the same order of magnitude in both Strains E and B. As shown in Tables 3 and 6, on the other hand, in both Strains E and B, only living cell counts for Sample 1 (containing more cells) determined by comparative method 2 using the calibration curve shown in Figure 1 were of the same order of magnitude as those determined by the inventive method and comparative method 1. With regard to Sample 2, both standard bacterial strains were substantially uncountable by comparative method 2 because they had cell counts less than  $10^3$  CFU/ml and thus an intensity equal to the blank value.

[0039] These results indicate that any sample containing more or less cells, including those uncountable by comparative method 2 (less than  $10^3$  CFU/ml), can be counted with order-of-magnitude accuracy in a short time by the inventive method.

#### Example 2: Determination of Contaminant (Living Cell) Counts in Cut Vegetables

[0040] Three types of commercially available cut vegetables were purchased. 100 ml of sterilized physiological saline was added to 10g of each cut vegetable, followed by stomaching treatment using a Stomacher (distributed by Organo Corporation). The stomached solution thus obtained was used as a sample.

##### (1) Inventive Method

[0041] The above samples were used and assayed according to the inventive method described in Example 1, except for a culture temperature of 30 °C and a culture period of 8 hours.

##### (2) Comparative Method 1

[0042] The above samples were used and assayed according to comparative method 1 described in Example 1, except for a culture temperature of 30 °C and a culture period of 2 days.

##### (3) Comparative Method 2

[0043] The above samples were used and assayed according to comparative method 2 described in Example 1, except that a cell-free sample prepared by filtration through a 0.22 µm filter was similarly assayed to obtain its luminescence intensity as a blank value.

##### (4) Results

[0044]

(i) The results obtained by the inventive method are shown in Table 7.



Table 7

Dilution factor	Sample 1		Sample 2		Sample 3	
	Intensity (RLU)	Growth	Intensity (RLU)	Growth	Intensity (RLU)	Growth
10	14381	Yes	Out of range	Yes	92932	Yes
100	1820	Yes	536332	Yes	7791	Yes
1,000	180	Yes	57266	Yes	634	Yes
10,000	80	Yes	6782	Yes	66	Yes
100,000	10	No	986	Yes	45	Yes
1,000,000	9	No	101	Yes	10	No
10,000,000	11	No	10	No	11	No
100,000,000	12	No	12	No	12	No
Blank value	10	No	10	No	9	No
Living cell count for Sample 1 (order of magnitude): $10^4$ CFU/ml						
Living cell count for Sample 2 (order of magnitude): $10^6$ CFU/ml						
Living cell count for Sample 3 (order of magnitude): $10^5$ CFU/ml						

(ii) The results obtained by comparative method 1 are shown in Table 8.

Table 8

Sample	Living cell count
Sample 1	$4.2 \times 10^4$ CFU/ml
Sample 2	$4.5 \times 10^6$ CFU/ml
Sample 3	$1.3 \times 10^5$ CFU/ml

(iii) The results obtained by comparative method 2 are shown in Table 9.

Table 9

Sample	Intensity	Living cell count
Sample 1	4689 RLU	uncountable
Sample 2	19028 RLU	$8.0 \times 10^6$ CFU/ml
Sample 3	5538 RLU	uncountable
Blank	4490 RLU	

[0045] In the three samples, all living cell counts determined by the inventive method in a short time (Table 7) and comparative method 1 (Table 8) were of the same order of magnitude. In comparative method 2, as shown in Table 9, Samples 1 and 3 were uncountable because the blank had a high value of 4490 RLU (unit of the intensity) and these two samples had the measured values nearly equal to the blank value. However, Sample 2 containing more contaminants had a significantly high measured value as compared with the blank value, thereby providing the result of the same order of magnitude as that of the inventive method.

[0046] These results indicate that any sample having various blank values, including those uncountable by comparative method 2 due to a high blank value, can be counted in a short time by the inventive method. The inventive method can also be used for the determination of contaminant count in foods, such as vegetables, without any problem.

#### Example 3: Determination of Contaminant (Living Cell) Counts in Meats

[0047] Three types of commercially available meats were purchased. 100 ml of sterilized physiological saline was added to 10g of each meat, followed by stomaching treatment using a Stomacher. The stomached solution thus obtained was used as a sample.

## (1) Inventive Method

[0048] The above samples were used and assayed according to the inventive method described in Example 1, except for a culture temperature of 30 °C and a culture period of 8 hours. CheckLite HS Plus (Kikkoman Corp.) was used as a luminescent reagent.

## (2) Comparative Method 1

[0049] The above samples were used and assayed according to comparative method 1 described in Example 1, except for a culture temperature of 30 °C and a culture period of 2 days.

## (3) Comparative Method 2

[0050] The above samples were used and assayed according to comparative method 2 described in Example 1, except that a cell-free sample prepared by filtration through a 0.22 µm filter was similarly assayed to obtain its luminescence intensity as a blank value. CheckLite HS Plus (Kikkoman Corp.) was used as a luminescent reagent.

## (4) Results

## [0051]

(i) The results obtained by the inventive method are shown in Table 10.

Table 10

Dilution factor	Sample 1		Sample 2		Sample 3	
	Intensity (RLU)	Growth	Intensity (RLU)	Growth	Intensity (RLU)	Growth
10	483366	Yes	32087	Yes	Out of range	Yes
100	28682	Yes	1893	Yes	143785	Yes
1,000	723	Yes	236	Yes	16190	Yes
10,000	56	Yes	68	Yes	1458	Yes
100,000	25	No	26	No	208	Yes
1,000,000	24	No	23	No	59	Yes
10,000,000	25	No	25	No	25	No
Blank value	24	No	10	No	25	No
Living cell count for Sample 1 (order of magnitude): 10 <sup>4</sup> CFU/ml						
Living cell count for Sample 2 (order of magnitude): 10 <sup>4</sup> CFU/ml						
Living cell count for Sample 3 (order of magnitude): 10 <sup>6</sup> CFU/ml						

(ii) The results obtained by comparative method 1 are shown in Table 11.

Table 11

Sample	Living cell count
Sample 1	4.0 × 10 <sup>4</sup> CFU/ml
Sample 2	1.5 × 10 <sup>4</sup> CFU/ml
Sample 3	2.8 × 10 <sup>6</sup> CFU/ml

(iii) The results obtained by comparative method 2 are shown in Table 12.

Table 12

Sample	Intensity	Living cell count
Sample 1	55928 RLU	uncountable
Sample 2	55160 RLU	uncountable

Table 12 (continued)

Sample	Intensity	Living cell count
Sample 3	57298 RLU	uncountable
Blank	53692 RLU	

[0052] In comparative method 2, as shown in Table 12, all three samples were uncountable because the blank had a very high value of 53692 RLU and all these samples had the measured values nearly equal to the blank value. On the other hand, all living cell counts determined by the inventive method and comparative method 1 were of the same order of magnitude in all three samples. Accordingly, even a sample uncountable by comparative method 2 due to a very high blank value can be counted in a short time by the inventive method. The inventive method is also useful for the determination of contaminant count in foods such as meat.

#### INDUSTRIAL APPLICABILITY

[0053] The present invention relates to a method for counting living cells in a sample by serially diluting the sample containing living cells with a growth medium for living cells as a diluent solution, culturing the resulting dilution in a short time, and then detecting intracellular ATP. The present invention also relates to an assay reagent kit used for the above method and a living cell counting device.

[0054] By using the present invention, the number of living cells in a sample can be determined with high sensitivity in a short time. In foods, particularly perishable foods, it is strongly desirable to count contaminants contained therein in a short time. Accordingly, the present invention can be used preferably for this purpose.

[0055] All publications cited herein are incorporated herein by reference in their entirety.

#### Claims

1. A method for counting living cells in a sample, which comprises the following steps (i) to (iv):

- (i) a first step for serially diluting the sample with a diluent solution;
- (ii) a second step for culturing each dilution from the first step;
- (iii) a third step for detecting intracellular ATP in each of the cultured dilutions from the second step; and
- (iv) a fourth step for calculating the number of living cells in the sample, based on the results of intracellular ATP detection from the third step.

2. The method according to claim 1, wherein the diluent solution used in the first step is a growth medium for living cells.

3. The method according to claim 1, wherein the diluent solution used in the first step is a growth medium for living cells which has been subjected to ATP decomposition.

4. The method according to claim 1, wherein ATP present in the dilution, excluding the intracellular ATP, is decomposed during or after the culture in the second step.

5. The method according to claim 1, wherein the intracellular ATP is detected in the third step using a luciferin-luciferase luminescent reaction.

6. An assay reagent kit used for the method according to claim 1, which comprises the following components (a) to (c):

- (a) a diluent solution,
- (b) an ATP-extracting reagent, and
- (c) an ATP-detecting reagent.

7. A living cell counting device used for the method according to claim 1, which comprises a device for sample dilution, a culture device for cell growth and a device for ATP detection.

8. The living cell counting device according to claim 7, wherein the device for ATP detection measures the intensity

of luminescence produced through a luciferin-luciferase luminescent reaction.

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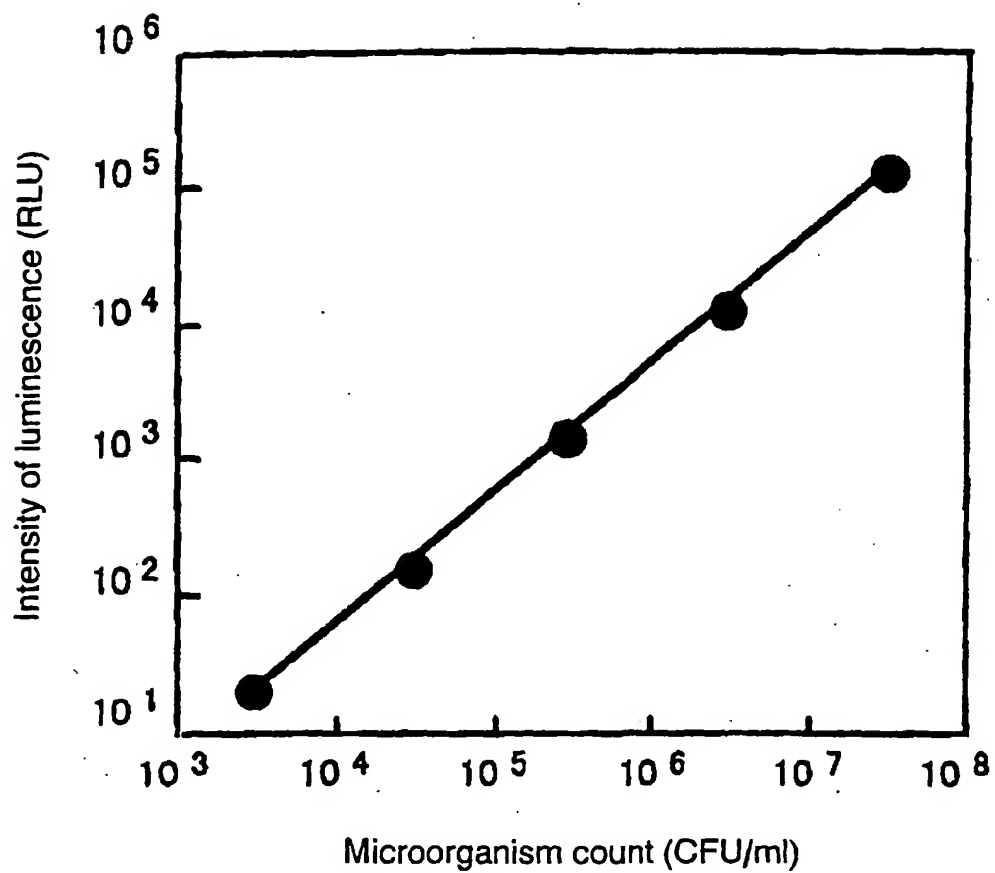
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Figure 1



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP99/06582

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl <sup>7</sup> C12Q1/66, C12Q1/06, C12M1/34		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl <sup>7</sup> C12Q1/00-C12Q1/66, C12M1/34		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) BIOSIS (DIALOG)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 10-191996, A (Toyo Ink Manufacturing Co., Ltd.), 28 July, 1998 (28.07.98) (Family: none)	1-8
Y	JP, 10-28599, A (Toyo Ink Manufacturing Co., Ltd.), 03 February, 1998 (03.02.98) & US, 5908751, A & EP, 803577, A1	1-8
Y	JP, 8-224096, A (SAPPORO BREWERIES LIMITED), 03 September, 1996 (03.09.96) & WO, 96/26290, A & EP, 757104, A1 & US, 5756303, A	1-8
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 21 February, 2000 (21.02.00)		Date of mailing of the international search report 07 March, 2000 (07.03.00)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)